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AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor

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The bicyclam AMD3100 (formula weight 830) blocks HIV-1 entry and membrane fusion via the CXCR4 co-receptor, but not via CCR5. AMD3100 prevents monoclonal antibody 12G5 from binding to CXCR4, but has no effect on binding of monoclonal antibody 2D7 to CCR5. It also inhibits binding of the CXC-chemokine, SDF-1α, to CXCR4 and subsequent signal transduction, but does not itself cause signaling and has no effect on RANTES signaling via CCR5. Thus, AMD3100 prevents CXCR4 functioning as both a HIV-1 co-receptor and a CXC-chemokine receptor. Development of small molecule inhibitors of HIV-1 entry is feasible.

The virus–cell fusion process is an attractive target for the development of drugs to prevent human immunodeficiency virus type 1 (HIV-1) infection of its target cells. HIV-1 enters CD4⁺ T cells by fusing at the plasma membrane after interacting with the CD4 molecule and one of several co-receptors. The first of these co-receptors to be identified was fusin[1], renamed CXCR4 to reflect its membership of the CXC family of chemokine receptors when its ligand was shown to be stromal-derived factor-1α (SDF-1α)[2–4]. CXCR4, an integral membrane protein with seven-transmembrane spanning segments, is the co-receptor for T-cell line-tropic (T-tropic) HIV-1 isolates[1]. These viruses, often also called syncytium-inducing (SI) viruses, are rarely transmitted but evolve in about half of HIV-1-infected people typically after 5 years of infection[5–7]. When they do, they are associated with a more rapid disease course, exemplified by an increased rate of CD4⁺ T-cell decline[5–7]. However, the most commonly transmitted HIV-1 strains have the macrophage-tropic (M-tropic), non-syncytium-inducing (NSI) phenotype[8]. The paramount co-receptor for these viruses is the CC-chemokine receptor, CCR5, although usage of CCR2b and CCR3 has also been documented[9–13]. Most primary, T-tropic strains are dual-tropic, in that they can use both CXCR4 and CCR5, although some use CXCR4 exclusively[14,15].

There are, therefore, multiple targets for inhibitors of the entry of HIV-1 isolates with different phenotypes. To date, two modified proteins that inhibit CCR5-mediated entry of M-tropic strains have been described[16,17]. These compounds, however, are derivatives of the natural CCR5-ligand, RANTES, made by peptide chemistry, and they have molecular masses around 8 kDa. It would, in principle, be desirable to have small molecule inhibitors that are amenable to bulk synthesis by traditional chemical techniques, and orally available. Here we show that AMD3100, a relatively simple molecule, is an inhibitor of HIV-1 entry, specifically via the CXCR4 co-receptor.

AMD3100 was previously described in the literature as JM3100 (refs. 18, 19); these designations are to the same molecule, but AMD3100 should now be adopted. AMD3100 (formula weight 830) is the octahydrochloride dihydrate of 1,1'-[1,4-phenylene-bis(methylene)]-bis-1,4,8,11-tetra-azacyclotetradecane. The core structure of the molecule has a molecular weight of 502, and is shown in Table 1 of ref. 19. The previously described JM2987 (refs. 18, 19) is the octahydrobromide dihydrate derivative of JM3100 (AMD3100). AMD3100 is the most active member of the bicyclam family of compounds; other, less potent, congeners differ solely in the linker bridging the two cyclam subunits[18].

The bicyclams were originally synthesized as part of a program to develop HIV inhibitors and were found to inhibit HIV-1 and HIV-2 infection at an early stage of the viral life cycle, before reverse transcription was initiated[18–21]. Several T-cell line-adapted (TCLA) and primary isolates of HIV-1 were shown in previous studies to be sensitive to AMD3100, as were some HIV-2 isolates but not simian immunodeficiency virus (SIV)[18–21]. An escape mutant of the NL4/3 molecular clone of HIV-1 LAI with decreased sensitivity to AMD3100 had multiple amino acid substitutions in gp120, many of them within, or in proximity to, the V3 loop[20,21]. Taken together, these studies suggested that AMD3100 could be acting to inhibit HIV-1 entry, by interacting either with the virus or its receptors. However, the bicyclams do not inhibit gp120-CD4 binding[18,19], which focused our attention on the co-receptors.

Inhibition by AMD3100 is early in the HIV-1 life cycle

A time-of-addition experiment using an *env* complementation assay of virus entry[9,10,22] was used to confirm that AMD3100 was active early in HIV-1 infection (Fig. 1). HIV-1 pseudotyped with the envelope glycoproteins of the TCLA clone, HxB2, was mixed with U87MG-CD4 cells expressing CXCR4 (obtained from Dan

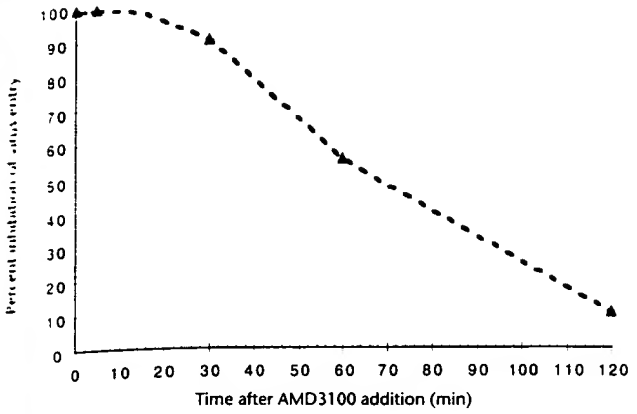


Fig. 1 Time course of AMD3100 action. U87MG-CD4-CXCR4 cells were mixed with HxB2 env-complemented HIV-1 at 37 °C (▲). AMD3100 was added at a final concentration of 10 ng/ml, either simultaneously with virus (0 min), or 5 min, 15 min, 30 min, 60 min or 120 min afterwards (see text). Inhibition of viral entry, measured by luciferase activity, is expressed as the inverse of the extent of entry in the absence of AMD3100 (defined as 100%). The data shown are representative of one of two independent experiments.

Littman)[23] for 2h at 37°C, and AMD3100 was added at different times during this period. Unbound virus and drug were washed away, and fresh drug was added back to the cultures. To have an antiviral effect, AMD3100 needed to be present soon after HIV-1 addition to the cells; the half-life of sensitivity to AMD3100 was 60-70 min (Fig. 1). This and previous results[18-21] were consistent with an effect of AMD3100 on virus entry.

AMD3100 blocks HIV-1 entry via CXCR4, but not via CCR5

To assess whether the action of AMD3100 was dependent on the co-receptor used by HIV-1 for entry, we again used the *env* complementation assay of entry in association with U87MG-CD4 cell lines that stably express either CXCR4 or CCR5 (Fig. 2). None of the viruses tested entered the parental U87MG-CD4 cells (data not shown). Entry into the CXCR4-expressing cells mediated by the envelopes of the TCLA strain HxB2 or the dual-tropic primary virus SF162-DBL was strongly inhibited by AMD3100 (IC_{90} values 0.01-0.1 nM) (Fig. 2*a*). Similar results were obtained with the pseudotypes expressing the envelopes of the TCLA virus NL4/3, and the dual-tropic primary virus DH123 (data not shown). However, the entry of M-tropic (ADA and JR-FL) or dual-tropic (SF162-DBL and DH123) viruses into the CCR5-expressing cells was insensitive to AMD3100 at concentrations up to 1200 nM (Fig. 2*b* and data not shown). Thus the identity of the co-receptor used, and not the virus, determines whether AMD3100 has an antiviral effect against HIV-1 in this assay; the same viruses (SF162-DBL and DH123) can be either sensitive or insensitive to the drug, depending upon the co-receptor with which they interact. Furthermore, the lack of effect of AMD3100 on HIV-1 entry and gene expression in the CCR5-expressing cells argues strongly against it having cytotoxic effects in the concentration range indicated. In addition, continuous exposure of either U87-CD4-CXCR4 or U87-CD4-CCR5 cells to 1200 nM AMD3100 for 3 days caused no change in cell viability, as determined by trypan blue exclusion. Because the luciferase signal in the *env* complementation assay requires only that the viral genome enters the cell and becomes integrated in the host DNA, the possibility that AMD3100 might also interfere with later events in the viral life (such as viral assembly)[19] is not excluded by this experiment. However, the finding that AMD3100 inhibits the productive infection of peripheral blood mononuclear cells (PBMCs) only by T-tropic, and not by M-tropic, primary HIV-1 isolates argues against AMD3100 having post entry effects[24].

To confirm the inhibitory action of AMD3100 in an independent test of *env*-mediated membrane fusion, we used the resonance energy transfer (RET) assay[9,25]. In this assay, HeLa-CD4 cells expressing the envelope glycoproteins of either the M-tropic virus JR-FL or the T-tropic virus LAI are mixed with PM1 cells, which have both the CCR5 and CXCR4 co-receptors. The lipids of the HeLa-CD4 and the PM1 cells are labeled with different fluorescent probes. Membrane fusion is measured by the amount of RET that occurs when these probes are brought into proximity. In this system, AMD3100 strongly inhibited membrane fusion mediated by the LAI envelope glycoproteins (IC_{90} <1 nM), but had no effect on JR-FL *env*-mediated fusion (Fig. 2*c*). The inhibitory range of AMD3100 in the RET fusion assay was comparable to that in the entry assay, and to the antiviral effects of AMD3100 in assays of cell-free HIV-1_{IIIB} replication[18,19]. However, syncytium formation in IIIB-infected cultures is relatively insensitive to AMD3100, with inhibitory effects occurring in the μM range[18,19]. Now that we have shown that AMD3100 targets the virus-cell fusion step, this discrepancy is no longer surprising; syncytium formation is known to be much less sensitive than virus entry to fusion inhibitors[26], probably because of the far greater number of interactions between the envelope glycoproteins and the cell that must be blocked[27]. The RET assay measures events taking place over a few hours, whereas syncytia form more gradually in a chronically infected culture and can involve the fusing together of hundreds to thousands of cells[28]. Furthermore, chronically infected cells express 10-100 fold more surface gp120/gp41 than the stable HeLa-Env cells used in the RET assay[25]. This assay is, therefore, much the more sensitive to inhibition by fusion antagonists.

AMD3100 interacts with CXCR4, but not detectably with gp120

The above experiments implicate the interactions between gp120 and CXCR4 as being the target for AMD3100. We first sought evidence of AMD3100 binding to gp120. Previous studies had shown that AMD3100 did not inhibit the binding of gp120 to CD4 or to a V3-loop monoclonal antibody[20,21]. However, monoclonal antibodies to several other gp120 epitopes can inhibit the interactions of gp120 with the CCR5 co-receptor[29,30], so we determined whether AMD3100 prevented the binding of these and other monoclonal antibodies to LAI (BH10) gp120, using a competition enzyme-linked immunosorbent assay (ELISA)[31]. The gp120 interaction of none of the 16 monoclonal antibodies tested (BAT-085, G3-136, 9284, CRA-1, 1C1, 133/290, 212A, A32, G3-508, G3-519, G3-299, G3-42, 2G12, 48d, 15e, 21h to 11 different epitope clusters[31]) or of the CD4-IgG molecule was significantly affected by AMD3100 (data not shown). This experiment strongly suggests (but does not formally prove) that AMD3100 interacts with a target other than HIV-1 gp120.

The development of monoclonal antibodies reactive with CXCR4 (12G5)[32] and CCR5 (2D7)[33] enabled us to test whether AMD3100 prevented monoclonal antibody–co-receptor interactions. Preincubation of both mitogen-stimulated PBMCs and of U87MG-CD4-CXCR4 cells with AMD3100 caused a dose-dependent inhibition of 12G5 reactivity. The lack of complete inhibition of 12G5 reactivity by AMD3100 may be due to the presence on cells of different CXCR4 species that do not react identically with 12G5, about which there has been speculation[34,35]. In contrast, AMD3100 did not affect the binding of 2D7 to either mitogen-stimulated

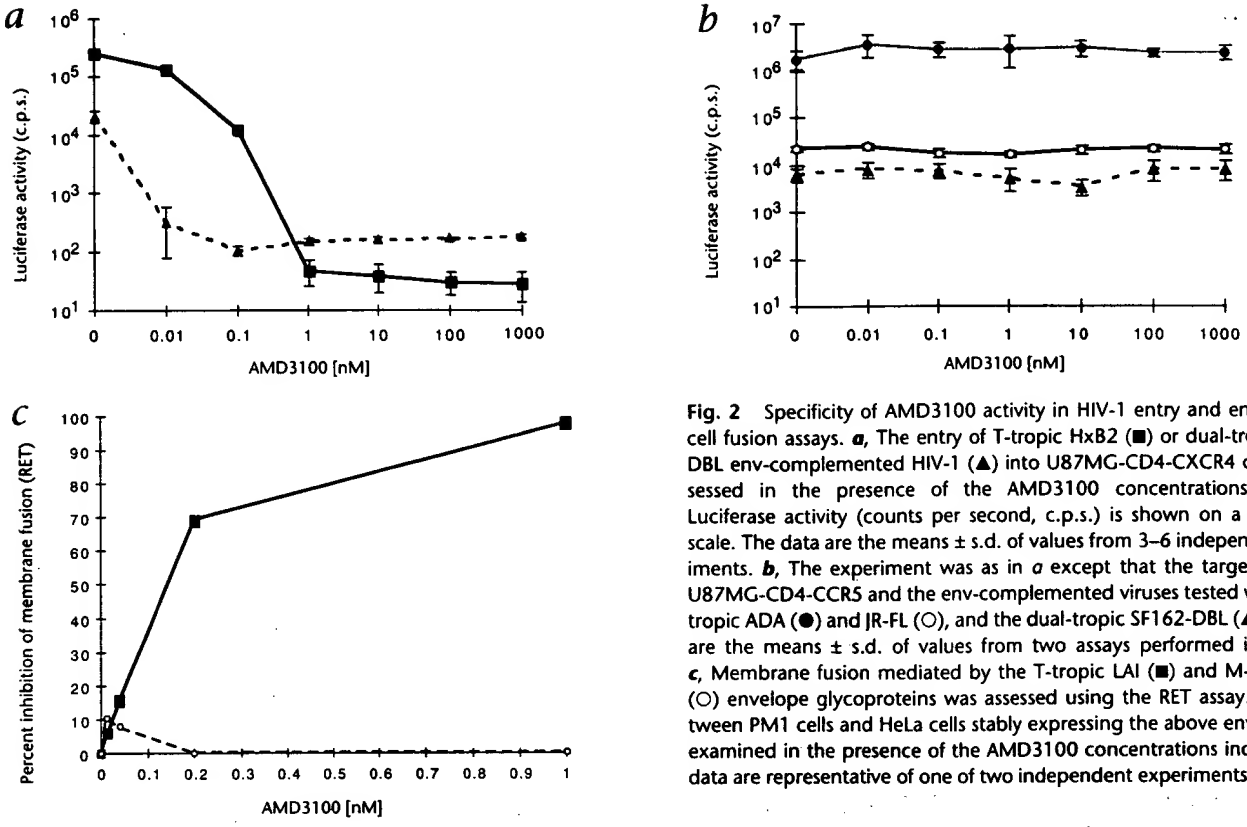


Fig. 2 Specificity of AMD3100 activity in HIV-1 entry and env-mediated cell fusion assays. ***a***, The entry of T-tropic HxB2 (■) or dual-tropic SF162-DBL env-complemented HIV-1 (▲) into U87MG-CD4-CXCR4 cells was assessed in the presence of the AMD3100 concentrations indicated. Luciferase activity (counts per second, c.p.s.) is shown on a logarithmic scale. The data are the means ± s.d. of values from 3–6 independent experiments. ***b***, The experiment was as in *a* except that the target cells were U87MG-CD4-CCR5 and the env-complemented viruses tested were the M-tropic ADA (●) and JR-FL (○), and the dual-tropic SF162-DBL (▲). The data are the means ± s.d. of values from two assays performed in triplicate. ***c***, Membrane fusion mediated by the T-tropic LAI (■) and M-tropic JR-FL (○) envelope glycoproteins was assessed using the RET assay. Fusion between PM1 cells and HeLa cells stably expressing the above env genes was examined in the presence of the AMD3100 concentrations indicated. The data are representative of one of two independent experiments.

PBMCs or U87MG-CD4-CCR5 cells (Fig. 3). The interaction of AMD3100 with CXCR4 was reversible, for 12G5 staining was fully restored after the cells were washed. Furthermore, the inhibition of 12G5 binding was not due to CXCR4 downregulation and internalization induced by AMD3100, since washing the cells at 4 °C permitted subsequent staining with 12G5 at the same temperature (data not shown). Under these conditions, CXCR4 recycling from internal pools to the cell surface would not be efficient. Thus, AMD3100 interacts at high affinity with CXCR4, to prevent both 12G5 binding and co-receptor activity.

Effect of AMD3100 on SDF-1α interactions with CXCR4

We next used a Ca^{2+} mobilization assay to test whether AMD3100 also affected the interactions of the CXC chemokine, SDF-1α, with CXCR4 (Fig. 4). We used 293T cells for these experiments because they have been well characterized for signal transduction studies. They were transiently transfected with CXCR4, because they do not endogenously express this receptor. AMD3100 did not itself cause signal transduction in 293T-CXCR4 cells even at 12 nM (Fig. 4*d*), but at 0.01 nM and 0.1 nM it, respectively, partially and completely inhibited Ca^{2+} signaling by SDF-1α (Fig. 4, *b* and *c*). In contrast, signaling by RANTES in CCR5-transfected 293T cells was not inhibited by 0.1 nM AMD3100 (Fig. 4*f*). The same concentration of AMD3100 also did not inhibit Ca^{2+} signaling by the macrophage inflammatory protein MIP-1β through CCR5 (not shown) or by carbachol or somatostatin through other G-protein-coupled receptors on 293T cells (Fig. 4). AMD3100 does not inhibit Ca^{2+} signaling nonspecifically, only when it is triggered via CXCR4.

Consistent with its effect on SDF-1α-induced signaling, AMD3100 completely inhibited the binding of ^{125}I-labeled SDF-1α to the MT-2 T-cell line, which endogenously expresses high levels of CXCR4. Half-maximal inhibition of binding occurred at approximately 0.01–0.1 nM AMD3100, and complete inhibition (to the same extent as caused by excess cold SDF-1α) was observed at 120 nM (Fig. 4*g*). Because the SDF-1α binding and signaling experiments were necessarily carried out using different cells, it is not clear whether there is any significance to the observation that 0.1 nM AMD3100 is sufficient to completely block Ca^{2+} signaling through CXCR4, while only blocking SDF-1α binding by about 50% (cf. Fig. 4, *b* and *g*). It is possible that signaling is inherently more sensitive to inhibition. Further studies will be required to resolve this.

Discussion

Taken together, our data strongly suggest that a small molecule, the bicyclam AMD3100, interacts with the CXC-chemokine receptor CXCR4 in such a way as to prevent its function as a HIV-1 co-receptor. Furthermore, the interaction of AMD3100 with CXCR4 inhibits that of the CXCR4-specific monoclonal antibody 12G5, and also prevents the CXCR4-ligand SDF-1α from binding to this receptor and activating signal transduction. Other results consistent with an interaction between AMD3100 and CXCR4 have also been described recently[24]. The lack of activity of AMD3100 on the interactions of HIV-1, the 2D7 monoclonal antibody and RANTES (regulated-upon-activation, normal T expressed and secreted) with CCR5 argues that it is specific for CXCR4 and not a general inhibitor of HIV-1 entry. That AMD3100 inhibits the entry of dual-tropic viruses (SF162-DBL and DH123) only when they use CXCR4 and has no effect on the same viruses entering via CCR5 (Fig. 2, *a* and *b*) demonstrates that AMD3100 does not interfere with early postfusion processes such as uncoating or integration; inhibition of these reactions would be independent of the co-receptor used for entry. In addition, AMD3100 inhibits *env*-mediated membrane fusion in the absence of other HIV-1 proteins (Fig. 2*c*), suggesting that the expression of gene products other than the envelope glycoproteins is not necessary for this compound to exert antiviral ac-

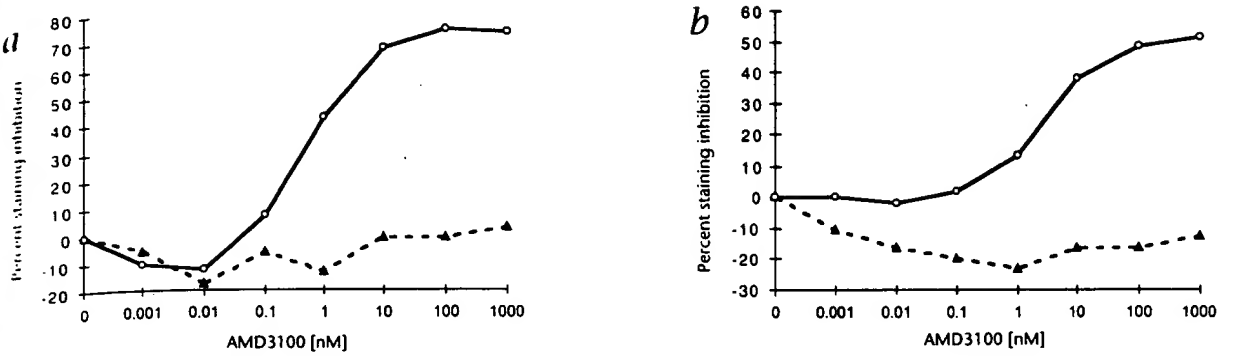


Fig. 3 Effect of AMD3100 on mAb reactivity with the CXCR4 and CCR5 co-receptors. *a*, Mitogen-stimulated PBMCs were stained for CXCR4 using the 12G5 mAb (○) or for CCR5 with the 2D7 mAb (▲), in the presence of the AMD3100 concentrations indicated. *b*, The experiment was as in *a*, except that U87MG-CD4-CXCR4 and U87MG-CD4-CCR5 cells were used with mAbs 12G5 and 2D7, respectively. For 12G5, the mean fluorescence intensity (MFI) on the U87-CD4-CXCR4 cells was 66.1, and on PBMCs, 67.3. For 2D7, the MFI on U87-CD4-CCR5 cells was 42.7, and on PBMCs, 19.3. The data are representative of one of two independent experiments.

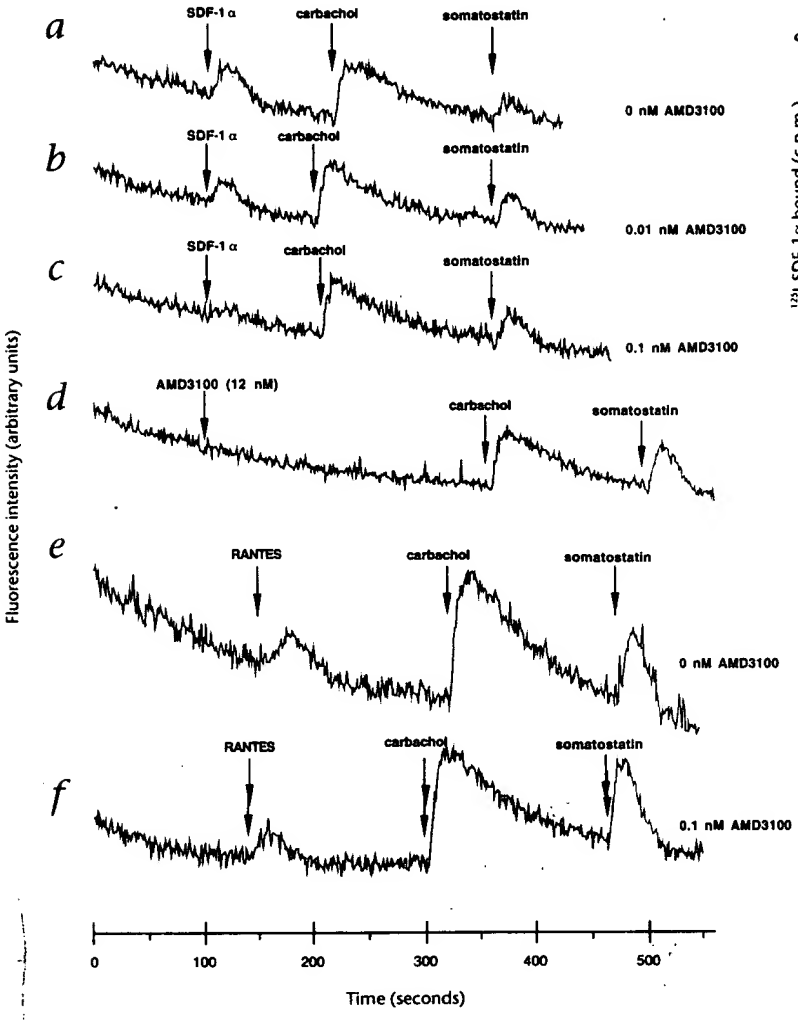
tivity. Furthermore, AMD3100-escape mutants of HIV-1_{IIIB} have amino acid sequence changes only in the gp120-encoding part of the *env* gene[20,21]. Although in the present study we used only assays of virus fusion and entry, which do not exclude an additional effect of AMD3100 late in the viral life-cycle (for example, during viral assembly), AMD3100 has no effect on the productive replication of M-tropic viruses in PBMCs (ref. 24). This argues against post entry effects of AMD3100 on the HIV-1 life cycle. A report that AMD3100 could inhibit the replication of a single HIV-1 strain in monocyte/macrophages[19] awaits confirmation.

How does AMD3100 work to prevent HIV-1 entry? Again, the fact that it inhibits the entry of dual-tropic viruses only via CXCR4 suggests (but does not prove) that its binding site is not located on the envelope glycoproteins. A case could be made that AMD3100 occupies a CXCR4-specific binding site somewhere on the envelope glycoprotein complexes of dual-tropic viruses without affecting a separate binding site on the same complexes for CCR5. Both co-receptor binding sites are, however, considered to be within the gp120 moiety[29,30,36,37], and we could find no evidence that any gp120 epitope was occluded by AMD3100. Whereas this does not formally prove that there can be no interaction between the HIV-1 envelope glycoproteins and AMD3100, it must be considered that AMD3100 also inhibits the binding of the 12G5 monoclonal antibody and of the SDF-1α ligand to CXCR4. This strongly suggests that AMD3100 interacts with CXCR4. Taking all these experiments together, the simplest explanation of the inhibitory effect of AMD3100 on HIV-1 entry is that it binds to CXCR4 to prevent the virus from doing so; the alternative explanation that it interacts with both a CXCR4-specific site on gp120 and with CXCR4 itself would, perhaps, be stretching the envelope.

How might AMD3100 interact with CXCR4? First, the various effects of AMD3100 could be readily reversed by washing the cells free of unbound drug, which implies that it does not covalently modify CXCR4. We could also find no evidence that AMD3100 causes CXCR4 down-modulation. The simplest explanation of all the data is that AMD3100 noncovalently binds to CXCR4 at a site that overlaps those used by HIV-1, 12G5 and SDF-1α and prevents the binding of these ligands (although we emphasize that we have not been able to prove that AMD3100 inhibits gp120 binding to CXCR4, for want of a suitable direct assay for gp120–CXCR4 interactions). Alternatively, the binding of AMD3100 to CXCR4 might cause a conformational change in the receptor that affects the integrity of the HIV-1, 12G5 and SDF-1α binding sites. In view of the small size of AMD3100 (formula weight 830) and the probably nonidentical natures of the HIV-1, 12G5 and SDF-1α binding sites[2,3,32,34,35,38–40], the latter may be the more likely possibility. For instance, AMD3100 might intercalate between the transmembrane helices of CXCR4 and change the tertiary structure of the receptor; ligand interactions with the seven-transmembrane helical receptors have been considered to involve the intra-helical domain[4,41,42]. An interaction of AMD3100 with the second extracellular loop of CXCR4 might also be able to account for its broad inhibitory activity. Detailed ligand-binding studies to discriminate between competitive and noncompetitive inhibitory mechanisms may resolve more precisely how AMD3100 acts, including whether there is any mechanistic significance to the observations that inhibition of SDF-1α-induced Ca^{2+} signaling occurs at AMD3100 concentrations that do not fully block SDF-1α binding.

AMD3100 was not designed to be an inhibitor of HIV-1 entry, so its action on CXCR4 is serendipitous. However, the present results do establish that a small molecule can potently inhibit the interaction of HIV-1 with a co-receptor, which suggests that drug-screening programs may also yield dividends. Indeed, in view of AMD3100's inhibition of SDF-1α-induced signaling, inhibitors of other chemokine receptors of medical relevance[4] might be similarly identifiable. Whether AMD3100 eventually has clinical utility will depend on how it performs in other stages of the drug development process. AMD3100 has been tested in SCID-hu mice, which showed that it is nontoxic and has antiviral activity but limited bioavailability[43]. But there are other issues, specific to HIV-1 and CXCR4, that may have an impact on its value as an anti-HIV-1 drug.

First, HIV-1 uses multiple co-receptors to enter its target cells[1,9–15], so inhibiting only one, CXCR4, may not be sufficient for a significant anti-viral effect *in vivo*. However, blocking CXCR4 may prevent the emergence of the more virulent, SI viruses that use this receptor[5–7,14,15], which would be beneficial. Second, we do not know what the consequences are of inhibiting CXCR4 function *in vivo*. Although the M-tropic HIV-1 co-receptor CCR5 is clearly dispensable for human health[44,45], this is not necessarily true of CXCR4. Indeed, knocking out the SDF-1 gene in mice creates a lethal phenotype[46]. Third, the high mutation rate of HIV-1 means that it may be able to evade a drug targeted at CXCR4, just as it does other single antiviral compounds. Already, a AMD3100 escape mutant of HIV-1 LAI has been made *in vitro*[20,21,24]. Studies are in progress to determine whether the resistant virus can still interact with CXCR4,



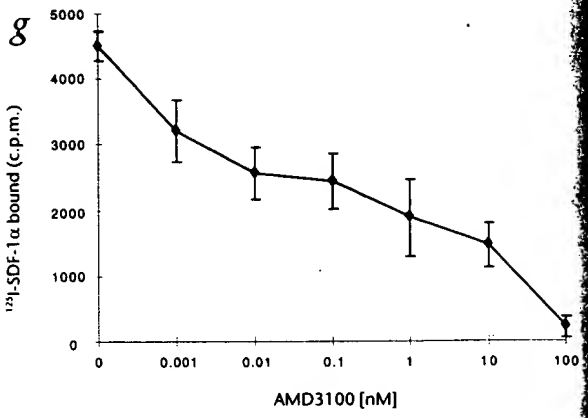


Fig. 4 SDF-1α-induced signaling via CXCR4 is blocked by AMD3100. *a–c*, 293T cells expressing CXCR4 were incubated for 15 min at room temperature with or without AMD3100 (0, 0.01 and 0.1 nM in *a*, *b* and *c*, respectively). Changes in the intracellular Ca^{2+} concentration in response to 100 nM SDF-1α were then measured, followed by secondary responses to ligands of other G-protein-coupled receptors (3 mM carbachol and 3 mM somatostatin). *d*, AMD3100 (12 nM) does not itself induce a Ca^{2+} flux or block the response to 3 mM carbachol or 3 mM somatostatin. *e and f*, Response of 293T cells expressing CCR5 to 100 nM RANTES in the absence (*e*) or presence (*f*) of 0.1 nM AMD3100. *g*, The amounts of ^{125}I-SDF-1α specifically bound to MT-2 cells in the presence of the indicated AMD3100 concentrations are recorded. Specific binding was estimated by subtracting from each data point the amount of nonspecific binding observed in the presence of excess unlabeled SDF-1α. The data are the means ± s.d. from three replicate determinations.

but in a AMD3100-insensitive manner, or whether it has evolved to use a different co-receptor. The escape mutant is no longer sensitive to inhibition by SDF-1α (ref. 24), perhaps suggesting the latter is the more likely possibility. Theoretically, some viruses may be able to use CXCR4 without interference by AMD3100, since there is a virus strain- and cell type-dependence to the *in vitro* antiviral activity of the 12G5 monoclonal antibody to CXCR4, for reasons that are not fully understood[34,35]. However, we have not observed any comparable restrictions on the inhibition of CXCR4-mediated entry by AMD3100.

Only more studies *in vitro* and *in vivo* could determine whether these potential hurdles are insurmountable obstacles to the development of AMD3100 as an antiviral drug. The identification here of AMD3100 as a small molecule inhibitor of the interactions of HIV-1 with a co-receptor is, however, a useful step towards the general development of HIV-1 entry antagonists, for it demonstrates proof of concept. The RANTES derivatives Met-RANTES and AOP-RANTES have potent activity, *in vitro*, against M-tropic strains that use CCR5 as a co-receptor[16,17]. It may eventually be possible to develop drug combinations able to block the use of multiple co-receptors. AMD3100 could be a valuable member of such a therapeutic cocktail.

Methods

Reagents. AMD3100 was obtained as described previously[18–21]. U87MG-CD4 cells stably expressing CXCR4 or CCR5 were kindly provided by Dan Littman[23]. The 12G5 anti-CXCR4 monoclonal antibody (mAb)[32] was purchased from PharMingen, Inc. (San Diego, CA) the 2D7 anti-CCR5 mAb (ref. 33) was a generous gift from Lijun Wu and Charles Mackay and SDF-1α was donated by Michael Siani [M.A. Siani *et al.* National Managed Health Care Congress (NMHCC): Chemokine receptors and host cell interaction. Abstr. 5, Baltimore, MD, 29–31 Jan. 1997; and ref. 47]. ^{125}I-labeled SDF-1α was purchased from DuPont-NEN, (Boston, MA). *Env* genes from the HIV-1 strains SF162-DBL and DH123 were provided by Cecilia Cheng-Mayer[48] and from Riri Shibata and Malcolm Martin[49], respectively. Carbachol was from Sigma Chemicals and somatostatin from Calbiochem (La Jolla, CA).

HIV-1 entry and cell fusion assays. U87MG-CD4 cells expressing either CXCR4 or CCR5 were seeded at 5×10^3 per well and cultured in 96-well plates for 20 h before infection with 10 ng of *env*-complemented HIV-1, which was prepared as described previously[9,10]. The cells were treated with various concentrations of AMD3100 for 15 min before infection. Recombinant HIV-1 viruses with the *nef* gene replaced by the firefly *luciferase* gene and bearing the ADA, JRFL, NL4/3, HxB2, SF162-DBL or DH123 envelopes were allowed to infect cells for 2 h at 37 °C, unless indicated otherwise. Cells were lysed 72 h postinfection and the lysates assayed for luciferase activity[9,10]. The RET assay of membrane fusion between HeLa-*env* cells and PM1 cells (a gift from Marvin Reitz) was performed as described previously,[9,25].

Monoclonal antibody inhibition assay. U87MG-CD4-CXCR4, U87MG-CD4-CCR5 or phytohemagglutinin plus IL-2-stimulated PBMCs (2.5×10^5) were washed, then incubated for 15 min on ice in 50 µl PBS/1%BSA/0.05% NaN_3 containing the indicated AMD3100 concentrations. mAbs 12G5 (25 µl at 10 µg/ml) or 2D7 (10 µl at 10 µg/ml) were then added for a further incubation for 45 min on ice. The cells were washed twice with cold PBS/0.1% NaN_3 and stained for 45 min on ice with 25 µl of RPE-conjugated goat anti-mouse IgG (1:50; IgG2a for 12G5, IgG1 for 2D7; Dako, Carpinteria, CA). After a final washing with PBS/0.1% NaN_3, the stained cells were fixed in

PBS/1% formaldehyde. Staining was quantified by flow cytometric analysis with a fluorescence-activated cell sorter (FACS; Becton Dickinson, San Jose, CA), and inhibition by AMD3100 was expressed as the percentage of the median fluorescence intensity recorded in the absence of AMD3100.

SDF-1α- or RANTES-induced Ca^{2+} fluxes. The pcDNA3.1 vectors (Invitrogen, San Diego, CA) containing the CXCR4 or CCR5 genes were transfected into 293T cells using lipofectamine. The cells were harvested 24 h later, washed, suspended in 12 ml of assay buffer (26 mM HEPES, 1 mM NaH_2PO_4, 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5.6 mM glucose, 0.2% BSA, 250μM probenicid) then incubated with 3.8 μM fluo-3,AM (Molecular Probes, Eugene, OR) for 1 h at 37 °C. For each reaction, 3×10^6 fluo-loaded cells were washed twice and incubated with or without AMD3100 for 15 min at room temperature. The fluorescence (λex 505 nm, λem 525 nm) from fluo-loaded cells in response to injection of 100 nM SDF-1α or 100 nM RANTES was monitored in a Hitachi F-2000 fluorescence spectrophotometer.

SDF-1α radioligand binding assay. The procedure used was a modification of that used for ^{125}I-MIP-1β binding[29]. MT-2 cells were washed twice and resuspended in cold binding buffer (RPMI 1640 medium containing 1% BSA, 25 mM HEPES and 0.05% NaN_3). AMD3100 or unlabeled SDF-1α was added to 1×10^6 cells, immediately followed by 0.05 nM ^{125}I-SDF-1α (2200 Ci/mmol) for 2 h at 37 °C. The extent of radioligand binding was determined as described previously[29].

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